Design, Synthesis and Biological Evaluation of Acridone Based Derivatives as Anti-Cancer Agents

SUMMARY FOR THE DEGREE OF

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Ph.D. Student

Ms. Tanuja T. Yadav

Research Supervisor

Dr. Mayur C. Yergeri

Shobhaben Pratapbhai Patel

School of Pharmacy and Technology Management

SVKM's Narsee Monjee Institute of Management Studies,

V.L. Mehta Road, Vile Parle (W),

Mumbai-400056

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1. Introduction

Cancer-one of the leading cause of mortality and morbidity in every region of the world. Worldwide, an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths were recorded in 2020. Female breast cancer has beaten lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancers[1].

Various strategies have been employed for the treatment of cancer which includes chemotherapy, surgery, hormone therapy, radiation therapy, targeted therapy, and stem cell transplant to get rid of the malignant tissues but each has its limits. So, usually, always a combination method is used [2]. At present, chemotherapy is the best approach used for the treatment of cancer. Despite the advances in treatment options and procedures, the clinical outcome is far from expectations. The toxicity of available drugs and the development of resistance over existing drugs increase the need for the development of newer anti-cancer agents with low toxicity and high efficacy. Hence, many scientists are struggling to develop newer chemotherapeutic molecules to discover safer, target-specific, and effective anti-cancer agents[3][4].

2. Literature Review

Protein kinases are intracellular enzymes that regulate the phosphorylation of proteins[5]. It is the largest group of kinases that phosphorylate at serine, threonine, and tyrosine residues and is involved in several signal transduction pathways[6]. There are mainly two types of protein kinases, serine/threonine kinases and other are tyrosine kinases. The serine/threonine kinase, AKT which is also referred to as Protein kinase B (PKB), is a key component of phosphatidyl-inositol-3-kinase (PI3K) that plays a crucial role in regulating cell growth, metabolism, proliferation, survival, migration, and angiogenesis[7][8]. It is activated by phosphorylation on Ser^{473} or Thr^{308} residues and it phosphorylates a variety of downstream protein substrates. The overexpression or hyper-activation of AKT has been observed in many cancers and is associated with increased cancer cell survival and proliferation. Thus, targeting AKT could provide an attractive approach for the design and development of small molecules as promising anti-cancer agents against cancer[9][10].

In this direction, the nitrogen-containing heterocycles have always shown promising anti-cancer activity and some of them are in clinical use [11]. Acridone is an interesting tricyclic *N*-heterocycle

containing a unique planar structure[12], can intercalate DNA base pairs[13], and acts as substrates for multi-drug resistance (MDR)[14] efflux pumps like P-gp[15] and breast cancer resistance protein (BCRP)[16]. It has been reported from the literature that this scaffold has the potential to interact with different biological targets such as DNA topoisomerase I/II [17][18], telomerase[19], dihydrofolate reductase (DHFR)[20], and protein kinases[21] (including cyclin-dependent kinases (CDKs)[22] and AKT)[23][24]. Hence, acridone scaffold has emerged as an important anti-cancer compound and drawn the attention of medicinal chemists to synthesize novel molecules[25].

Many research scientists tried to design and synthesize acridone-based scaffolds which would have the ability to display anticancer activity by interacting with multiple targets. Among them, some studies discovered that substituted acridones have the ability to inhibit AKT kinase. For example, Houghton et al. (2006), patented the 2-Br/Cl-N^{10}-substituted acridone compounds as AKT inhibitors and revealed that these molecules can inhibit AKT phosphorylation and kinase activity. Among them, most of the N^{10}-butyl substituted 2-chloroacridones (**1a**-**1e**) were found to be effective against AKT as compared to N^{10}-butyl substituted 2-bromoacridones except (**2a** and **2b**) at < 5 µM concentration[23]. Murahari et al. (2017), have identified and reported the series of acridone-pyrimidine hybrids as potent AKT inhibitors. This study found that these hybrid molecules (**3a**-**3c**) showed a good cytotoxicity profile against A-549 (lung), MCF-7, and MDA-MB-231 (breast) cancer cell lines and exhibited anti-cancer activity partly by DNA intercalation. Further, mechanistic studies revealed that they have the potential to inhibit AKT kinase activity and induce apoptosis. *In vitro* AKT kinase assay identified compounds (**3a**-**3c**) as potent AKT inhibitors at IC_{50} less than 10 nM[24].

On the other hand, there are several AKT inhibitors are under clinical investigation, including ATP-competitive pan-AKT inhibitor, AZD5363, AstraZeneca's invention, which is also known as Capivasertib (**Fig. 1**) [26]. It inhibits all AKT isoforms (AKT1/AKT2/AKT/3) with a potency of <10 nM and inhibited phosphorylation of AKT substrates in cells with a potency of approximately 0.3- 0.8 µM[27]. Currently, this drug is in phase III clinical trial with two different combinations. One is with Fulvestrant in metastatic hormone receptor-positive/human epidermal growth factor receptor 2 Negative (HR+/HER-) breast cancer (CAPItello-291) and the second one is in combination with Palbociclib + Fulvestrant in HR+/HER-advanced breast cancer (CAPItello-292) [26].

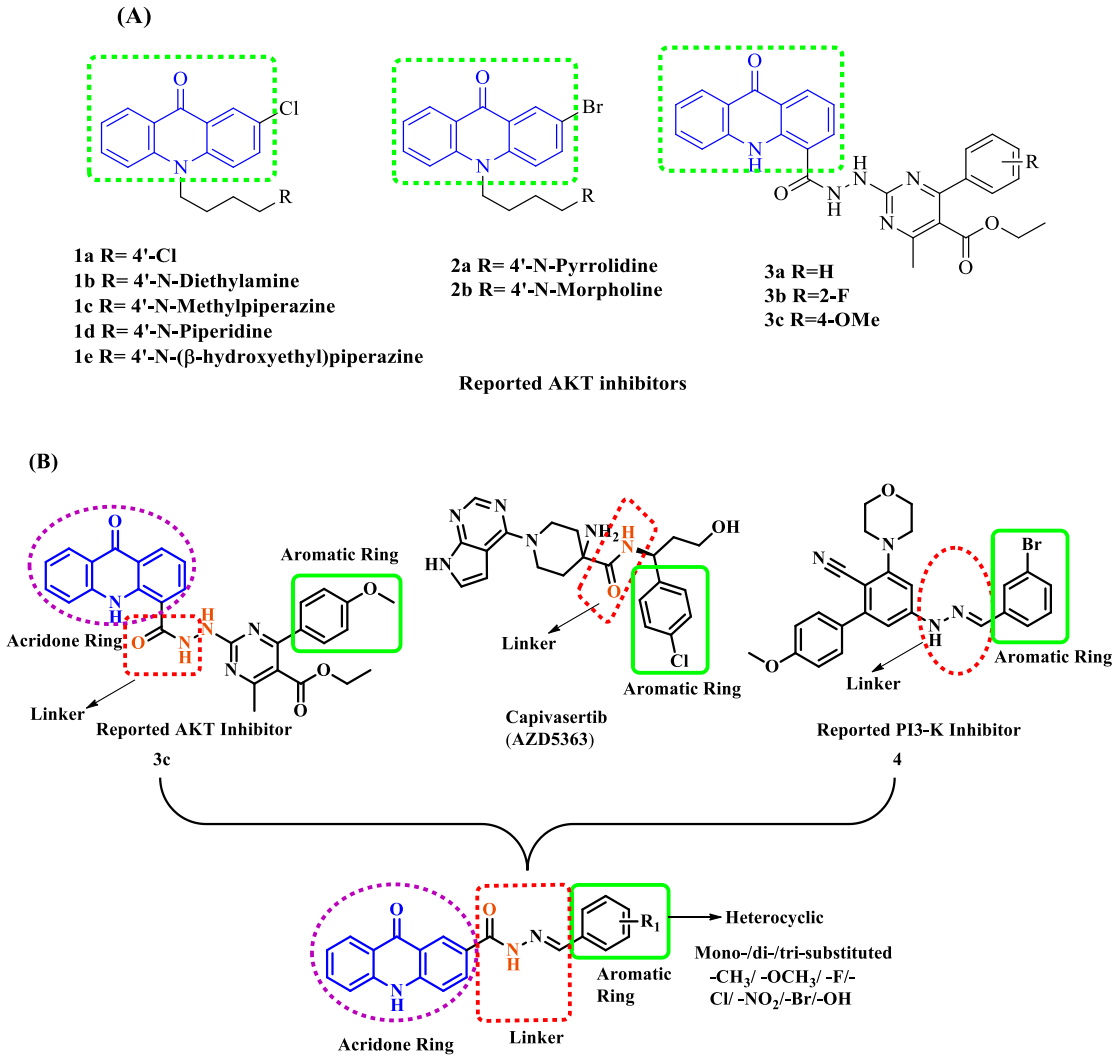


Figure 1: (A) Structures of acridone derivatives as AKT inhibitors (B) Design approach of acridone-2-carbohydrazide derivatives

Helwa et al. (2020), designed, synthesized, and evaluated 6-morphilino-pyrimidine derivatives as PI3-K inhibitors. Here, the 6-morphilino-pyrimidine scaffold was linked with aromatic rings through a hydrazinyl linker. All the synthesized compounds were screened for their cytotoxicity against a panel of NCI-60 cells. Among them, compound **4** showed the most potent anti-proliferative activity against the leukemia SR cell line with IC_{50} of 0.76 µM. PI3-K kinase inhibition assay found that compound **4** was found to be a promising inhibitor with IC_{50} of 11.73, 6.09, and 11.18 µM against (α), (β), and (γ) of PI3K class IA respectively. The presence of

hydrazinyl linker and *m*-substitution on phenyl ring displayed an increase in anti-cancer activity against a panel of cell lines[28].

Based on previous studies of acridone containing carbohydrazide linker and interesting activity of hydrazinyl compounds, we have designed acridone-2-carbohydrazide derivatives, characterized and screened for *in vitro* anticancer activity against human cancer cell lines A-549 (lung), MCF-7 and MDA-MB-231 (breast), and A-431 (skin) respectively.

3. Rationale

Despite the comprehensive, and target-based research on the development of acridone derivatives, not even a single molecule has entered the market. The planar and tricyclic aromatic ring of acridone showed a broad spectrum of biological utilization. Many derivatives of acridone were recommended for clinical studies, amongst them Acronycine derivatives went through phase I of clinical trials against various solid tumors, triazoloacridone has been selected for extended preclinical studies and imidazoacridone is currently in phase II clinical trials under the name Symadex TM®. Moreover, MDR inhibitory activity shown by the combination of Elacridar with Topotecan and Doxorubicin exhibited MDR inhibitor activity in Phase I clinical trials against solid tumors. Acridone's future discovery deceits in the designing of new molecules with more selectivity and less inherent toxicity. Recent research has opened new directions for the design and development of new and more potent inhibitors of Protein kinases, AKT enzyme, and P-gp as anticancer drugs. SAR study of acridone derivatives revealed that N^{10}-substituted acridone derivatives, multi- substituted acridone analogs showed increased cytotoxicity. Thus, the acridone moiety can still serve as a lead structure and can be further modified to increase the specificity toward cancer cells.

4. Objectives

Objectives of the proposed work are as follows:

1. To design novel scaffold based on reported molecules and the availability of modelling from the literature.
2. To synthesize and characterize the ligands.

3. To evaluate the synthesized ligands by *in vitro* assays to determine its potency. Based on the assay results the promising molecules will be subjected to extensive structure-activity relationship studies and next generation of molecules will be designed to improve biological activity.
4. To evaluate the promising molecules for various mechanism studies.

5. Experimental work

5.1 Chemical Synthesis and Characterization of acridone derivatives

5.1.1 Synthesis of acridone-2-carbohydrazide derivatives from scheme I

Synthetic Scheme I:

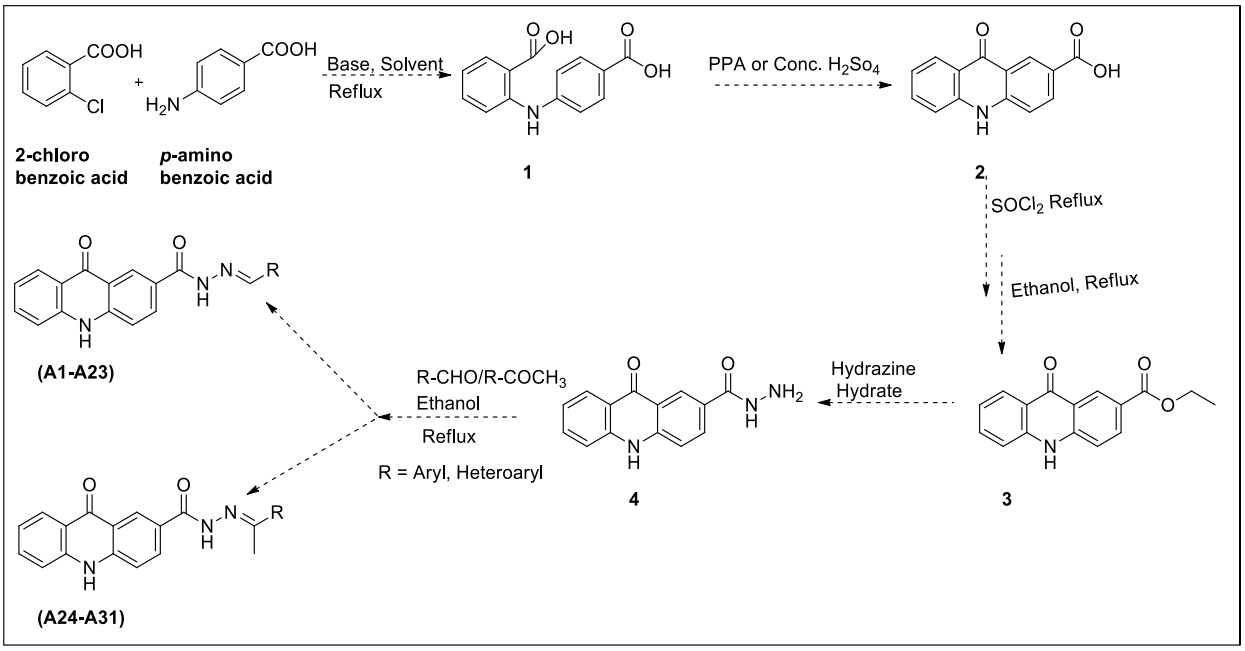


Figure 2: Synthetic scheme for Acridone carbohydrazide derivatives

General procedure for compounds A1-A23 and A24-A31

To a suspended solution of Int 4 (0.2 g, 1 equiv) in ethanol (10 mL) with glacial acetic acid (1 mL), aldehydes/ketones (1.5 equiv) were added dropwise and the reaction mixture was refluxed for 12- 14 h. After completion of the reaction, the mixture was allowed to cool at RT and then

concentrated to dryness. Cold water was added to the reaction mixture, filtered, and the crude product was recrystallized from a suitable solvent to give the desired products (**A1-A23**) and (**A24-A31**) (Scheme 1).

- **Synthesis of acridone-2-carbohydrazide derivatives (A1-A23)**

Reaction:

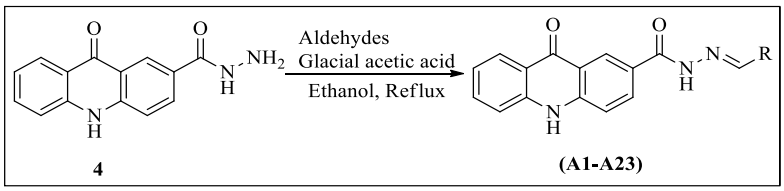


Table 1: Acridone 2-carbohydrazide derivatives (A1-A23) with Mass Data

Sr. No.	R	Mass (ESI/MS)	Observed Mass $[M+H]^+$	Yield (%)
1	-Phenyl	341.11	342.15	67.20
2	-Styryl	367.13	368.20	41.36
3	-2-Hydroxyphenyl	357.11	358.15	38.36
4	-4-Fluorophenyl	359.10	360.20	71.20
5	-4-Bromophenyl	420.02	420.05 & 422.05	47.82
6	-4-Methoxyphenyl	371.12	372.20	52.86
7	-4-Chlorophenyl	375.07	376.15 & 378.15	42.15
8	-4-Methylphenyl	355.13	356.20	78.80
9	-2-Fluorophenyl	359.10	360.20	70.20
10	-2-Chlorophenyl	375.07	376.15 & 378.15	40.35
11	3-Methoxyphenyl	371.12	372.20	37.86
12	-2-Nitrophenyl	386.10	387.15	32.35
13	-3,4-dimethoxyphenyl	401.13	402.25	53.86
14	-2,5-dimethoxyphenyl	401.13	402.25	52.48
15	-3,4-Dihydroxyphenyl	373.11	374.20	43.64
16	-4-Hydroxy 3-methoxyphenyl	387.12	388.20	41.95
17	-3,4,5-Trimethoxyphenyl	431.15	432.15	52.18
18	-4-Hydroxy 3,5-dimethoxy phenyl	417.13	418.25	59.76
19	-4-Benzyloxyphenyl	447.16	448.20	41.72

20	-4-Dimethylaminophenyl	385.16	385.25	48.59
21	-4-Cyanophenyl	366.11	367.15	46.52
22	-4-Pyridyl methylene	342.11	343.15	53.56
23	-2-Furanyl	331.11	332.15	47.35

- **Synthesis of acridone-2-carbohydrazide derivatives (A24-31)**

Reaction:

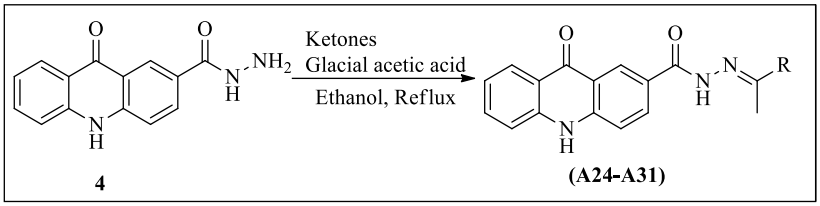


Table 2: Acridone 2-carbohydrazide derivatives (A24-A31) with Mass Data

Sr. No.	R	Mass (ESI/MS)	Observed Mass $[M+H]^+$	Yield (%)
1	-Phenyl	355.13	356.20	57.24
2	-4-Hydroxyphenyl	371.13	372.20	50.86
3	-4-Methoxyphenyl	385.14	386.20	68.84
4	-4-Methylphenyl	369.15	370.20	56.80
5	-4-Fluorophenyl	373.12	374.20	58.24
6	-4-Chlorophenyl	389.15	390.20 & 392.20	39.85
7	-4-Nitrophenyl	401.12	401.20	29.85
8	-2,4-Dichlorophenyl	424.05	424.20 & 426.20	32.46

A total of **31 compounds** were synthesized from scheme I (**Fig. 2**) and evaluated further by *in vitro* cytotoxicity assay.

5.1.2 Synthesis of Acridone-*N*-acetamide derivatives from scheme II

Synthetic Scheme II:

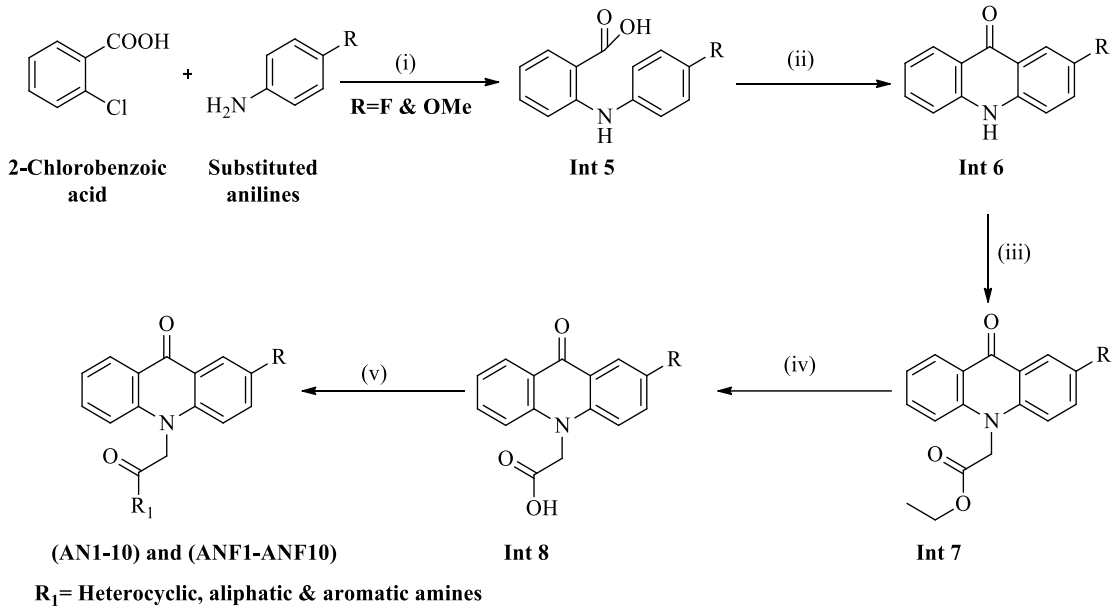


Figure 3: Synthesis of acridone-*N*-acetamide derivatives Reagents and conditions: i) Cu, K_2CO_3, Isoamyl alcohol, Reflux ii) PPA, 100°C iii) Ethylbromoacetate, K_2CO_3, DMF, 0°C-RT iv) NaOH, MeOH: H_2O (1:1) v) EDC.HCl, HOBt, DIPEA, DMF,0°C-RT

General procedure for synthesis of Acridone-*N*-acetamide derivatives (AF-1 to AF-10 and **AN-1 to AN-10)**

To a stirred solution of **Int 8** (0.12 g, 1 eq.) and substituted aromatic/aliphatic/heterocyclic amine (1.1 eq.) in (DMF) (10 mL) at 0°C, was added EDC.HCl (0.13 g, 1.5 eq.), HOBt (0.08 g, 1.5 eq.), and DIPEA (0.1 g, 3.0 eq.), were added. The reaction was stirred for 16-20 hrs. The reaction was monitored by TLC and after completion of the reaction, precipitates were collected by filtration and then washed with cold water and DCM. The crude product was purified by using column chromatography (DCM: methanol) (9.5:0.5) to get desired compound **(AF-1 to AF-10** and **AN-1 to AN-10).**

Reaction:

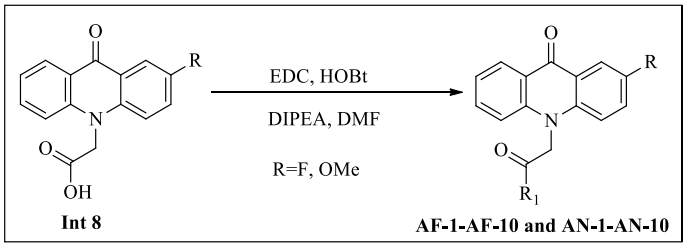


Table 3: Acridone N-acetamide derivatives with Mass Data

Sr. No.	Comp. Code	R	R_1	Mass (ESI/MS)	Observed Mass $[M+H]^+$	Yield (%)
1	AF-1	-F	-NH-thiazolyl	353.14	354.15	57.26
2	AF-2	-F	$-NH(CH_2)_2-OCH_3$	328.12	329.12	42.56
3	AF-3	-F	$-NH(CH_2)_2-N(CH_3)_2$	341.15	342.20	48.35
4	AF-4	-F	$-NH(CH_2)_2-N(C_2H_5)_2$	369.15	370.20	51.27
5	AF-5	-F	-Morpholinyl	340.12	341.15	57.62
6	AF-6	-F	-Piperidinyl	338.15	339.15	62.51
7	AF-7	-F	$-NH-C_6H_5$	346.11	347.15	49.76
8	AF-8	-F	$-NH-4'F-C_6H_5$	364.10	365.20	47.61
9	AF-9	-F	$-NH-4'Me-C_6H_5$	360.13	361.20	51.43
10	AF-10	-F	$-NH-4'OMe-C_6H_5$	376.13	377.20	47.35
11	AN-1	-OMe	-NH-thiazolyl	365.05	366.05	51.23
12	AN-2	-OMe	$-NH(CH_2)_2-OCH_3$	340.10	341.10	41.28
13	AN-3	-OMe	$-NH(CH_2)_2-N(CH_3)_2$	353.10	354.10	38.66
14	AN-4	-OMe	$-NH(CH_2)_2-N(C_2H_5)_2$	381.15	382.05	52.31
15	AN-5	-OMe	-Morpholinyl	352.08	353.10	47.82
16	AN-6	-OMe	-Piperidinyl	350.10	351.16	57.18
17	AN-7	-OMe	$-NH-C_6H_5$	359.13	359.20	52.70
18	AN-8	-OMe	$-NH-4'F-C_6H_5$	376.12	376.20	47.82
19	AN-9	-OMe	$-NH-4'Me-C_6H_5$	373.35	373.20	54.25
20	AN-10	-OMe	$-NH-4'OMe-C_6H_5$	389.14	389.20	40.35

A total of **20 compounds** were synthesized from scheme II (**Fig. 3**) and evaluated further by *in vitro* cytotoxicity assay.

5.1.3 Synthesis of *N*-substituted Acridone-2-carboxamide derivatives

Synthetic Scheme III:

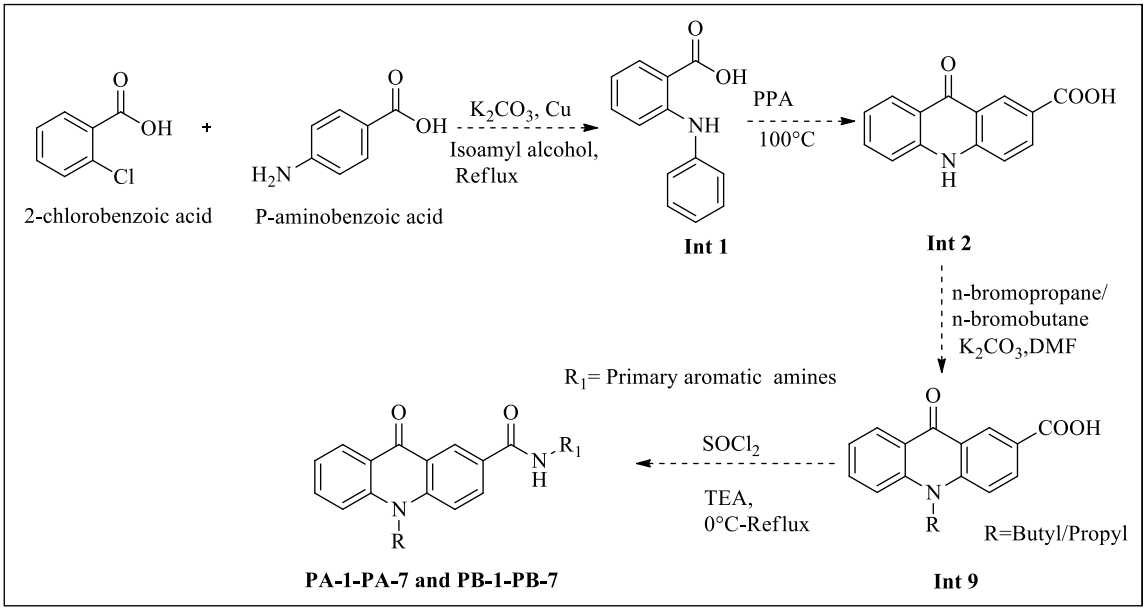


Figure 4: Synthetic scheme for acridone-2-carboxamide derivatives

Reaction:

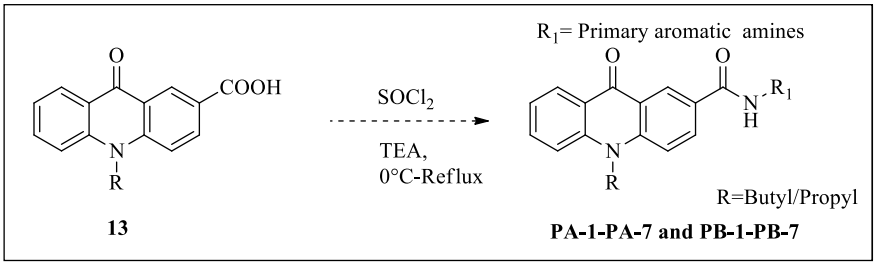


Table 4: Synthesized carboxamide derivatives

Sr. No.	Comp. Code	R	R1	Mass (ESI/MS)	Observed Mass	Yield (%)
1	PA-1	Propyl	-Phenyl	356.42	357.22	57.21
2	PA-2	Propyl	-4-Methylphenyl	370.44	371.21	41.38
3	PA-3	Propyl	-4-Methoxyphenyl	386.44	387.22	38.79
4	PA-4	Propyl	-4-Fluorophenyl	374.41	375.21	43.21
5	PA-5	Propyl	-4-Chlorophenyl	390.86	391.20 & 393.20	40.82
6	PA-6	Propyl	-4-Nitrophenyl	401.41	402.20	52.86
7	PA-7	Propyl	-Azobenzenephenyl	460.52	461.22	42.15
8	PB-1	Butyl	-Phenyl	370.44	371.20	53.24
9	PB-2	Butyl	-4-Methylphenyl	384.47	385.20	48.26
10	PB-3	Butyl	-4-Methoxyphenyl	401.43	401.20	29.56
11	PB-4	Butyl	-4-Fluorophenyl	388.43	389.20	50.86
12	PB-5	Butyl	-4-Chlorophenyl	404.13	405.20 & 407.20	38.84

13	PB-6	Butyl	-4-Nitrophenyl	415.15	416.20	46.80
14	PB-7	Butyl	-Azobenzenephenyl	474.38	475.25	41.23

A total of **14 compounds** were synthesized from scheme III (**Fig. 4**) and evaluated further by *in vitro* cytotoxicity assay.

5.2 Biological evaluation of synthesized compounds

5.2.1 *In vitro* cytotoxicity testing by sulforhodamine B (SRB) Assay

The anti-cancer activity of synthesized acridone compounds were assessed using SRB assay on human lung cancer cells (A-549), human skin cancer cells (A-431), human breast cancer cells (MCF-7 & MDA-MB-231) and normal murine fibroblast cell (NIH/3T3). Briefly, stock solutions of synthesized compounds and doxorubicin (positive control) were prepared to obtain a stock solution of 10 mM concentration. Dilutions (10 and 25 μM) were made from the stock solution. Diluted cell suspension (approximately 5,000 cells) was added to each well of the 96-well plate. After 24 hrs, different concentrations of test drugs were added and plates were then incubated at 37°C for 3 days in a 5% CO2 atmosphere and a microscopic examination was performed after 24 hrs interval. The cells were fixed using ice-cold trichloroacetic acid (TCA) for 1 hr at 4°C added to each well. And then washed using distilled water to remove excess TCA and allowed to dry in the air. After 2 hrs, the 50 μL of SRB solution was added to each well and allowed to stain at room temperature for 30 mins. The plate was washed with 1% v/v acetic acid to remove the unbound dye and allowed to dry in the air. About 100 μL of 10 mM Tris Base (pH 10.5) was added to each well and the plates were gently shaken for 5 mins on a shaker platform to extract the bound SRB. The absorbance was measured using a microplate reader at a wavelength of 510 nm[29].

5.2.2 *In-vitro* AKT kinase inhibition assay:

Compounds were evaluated for their ability to inhibit AKT kinase activity by using the Enzyme assay kit. According to the manufacturer's instructions, all the reagents and working standards were prepared. The cells were treated with selected acridone derivatives, at different concentrations, and the reported AKT inhibitor was used as a reference compound. The cell lysates were prepared and assayed for the estimation of protein concentration by using a Pierce BCA protein assay kit.

The AKT substrate microtiter plate was soaked with kinase assay dilution buffer at RT for 10 mins. A volume of 30 µl of each of the diluted solutions (control, standard, and samples) was added to each empty well in duplicate. The reaction was initiated by adding 10 µl of diluted ATP and was incubated for 90 mins at RT. Then 40 µl of phospho-specific substrate antibody, as well as the diluted secondary antibody was added and incubated for a given time. Then 60 µl of 3,3',5,5-tetramethylbenzidine (TMB) was added into each well to develop color in the wells. After sufficient color development, 20 µl of stop solution was added to the wells. Optical density was recorded with a plate reader at 450 nm. Data were analyzed and results are expressed as IC_{50} values using different concentrations [30][31].

5.2.3 Quantitative estimation of phosphorylated (p-AKT Ser^{473}) or total AKT protein

All the reagents and samples were prepared as instructed in the manual. Briefly, 100 µl of cell lysates were added into wells of ELISA kits and incubated for 2.5 h at RT. Then, discarded these lysates and washed with buffer 4 times. 100 µl of primary antibodies (p-AKT and Total AKT) were added to each well and kept for 1h at RT. Then secondary antibody solutions (1X HRP-Streptavidin, 100 µl) were added to measure phosphorylated or total protein in the cell lysates. The TMB substrate reagent (100 µl) was added followed by 50 µl of a stop solution. Immediately read at 450 nm. The activation status of AKT signaling proteins was evaluated according to the formula that the OD value of p-AKT/the OD value of total AKT[32].

5.2.4 Cell cycle analysis assay

Flow cytometric analysis was used to observe the cell cycle progression of MCF-7 cells with or without treatment of synthesized compounds. The standard PI staining method was used to determine the effect of selected compounds on the cell cycle[33][34]. MCF-7 cells were seeded at a density of (2.5×10^5/well) in a 6-well plate and incubated at 37 ℃ for 24 h. Subsequently, media was removed and the cells were treated with compounds at their IC_{50} concentration for 24 h. Then cells were collected and washed twice with cold PBS, fixed in ice-cold 70% (v/v) ethanol overnight at 4 ºC. Again, cells were washed with PBS, re-suspended with 100 µg/ml RNase, then the DNA of the cells was stained with 50 µg/ml PI, and incubated for 10 mins in the dark. The acquisition and analysis of data were performed by using a flow cytometer of the FACS Central Facility at IIT Bombay [35].

5.2.5 Apoptosis Assay

The Annexin V/ (PI) double staining method was used to carry out apoptosis assay with help of flow cytometry [36]. Annexin-V-FITC/ PI apoptosis detection kit was used for the determination of apoptosis. MCF-7 cells were plated to a 6-well plate with a density of (2.5×10^5/well) and incubated for 24 h. Then cells were treated with the selected compounds at 5 µM concentrations for 24 h. Cells were trypsinized and washed with PBS, then re-suspended in 1X binding buffer at a concentration of (1.0×10^6 cells/ml). From that, 100 µl of the test solution at a concentration of (1×10^5 cells) treated with the mixture of Annexin V-FTIC (5 µl) and PI (5 µl) and incubated in the dark for 10-15 mins at RT. 400 µl of binding buffer was added and cells were re-suspended again just before the flow cytometry analysis and performed using FACS Caliber flow cytometer at IIT Powai[37][38].

5.3 Computational Studies

5.3.1 Molecular Docking

To identify binding interactions of synthesized molecules within the binding site of the target protein i.e.; AKT, Autodock Vina software was used [39]. The target protein was retrieved from the RCSB protein data bank, and a protein (PDB ID: 4GV1) with zero mutation and a resolution of less than 5Å was selected. This protein consists of a co-crystallized ligand AZD5363. A docking study was performed as per the reported method [40]. Preparatory steps were carried out for receptor and ligands using Auto Dock Tools. In this step, water molecules, hetero groups, and unwanted chains are eliminated so that the protein structure is optimized. Then prepared protein was subjected to Grid Generation under AutoDock Tools and a grid box was generated at the active site of a co-crystalline ligand with the centered dimension cubic grid box of 30Å× 30Å × 30Å. Once the grid is set, the protein is saved in PDBQT format. The co-crystallized ligand AZD5363 and the acridone derivatives were drawn using ChemDraw software. Ligand structures were converted to the proper format (PDBQT) for docking. The docking protocol was run (n=9 for each ligand) using AutoDock Vina and the output was obtained [41]. The docking procedure was validated, wherein the overlay method, the crystallized ligand AZD5363 from the AKT was sketched and re-docked into the active site of AKT (PDB ID: 4GV1). The docked conformation overlapped over the crystallized bioactive conformation of the ligand available in the AKT

complex. The parameters utilized in the docking study were validated. The molecular docking was performed to observe possible protein-ligand interaction and binding affinity. The binding score for each of the structures was determined in kcal/mol. Results of the docked ligand complexes of AKT were analyzed in BIOVIA/Discovery Studio 2017R2. 2D "Receptor-Ligand Interactions" modules were used to check various types of binding interactions [42].

5.3.2 *In silico* ADMET studies

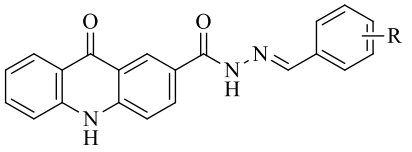
For the prediction of ADME parameters (Absorption, distribution, metabolism, and elimination) of molecules, Swiss-ADME [43], a web tool was used. It calculates the physicochemical and significant pharmacokinetic properties of molecules. In addition to that, toxicity data was predicted by TEST and Protox-II [44] online software. Oral rat LD50 (mg/kg) values were predicted for potential molecules.

6. Result and Discussions

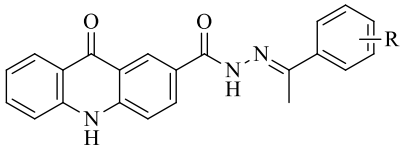
6.1 Chemistry of target acridone derivatives

6.1.1 Chemistry of acridone -2-carbohydrazides

Synthesis of acridone starts with the Ullmann condensation of halo benzoic acid and *p*-amino benzoic acid in the presence of copper as a catalyst. Cyclization with polyphosphoric acid yielded acridone-2-carboxylic acid. Further esterification and treatment with hydrazine hydrate gave carbohydrazide of acridone in the presence of solvent ethanol. The final compounds were synthesized by linking the carbohydrazide of acridone with different substituted aldehydes/ ketones in ethanol in the presence of acetic acid which yielded two series, one with substituted benzaldehydes, (**A1-A24**) and the other with substituted acetophenones (**A25-A31**).

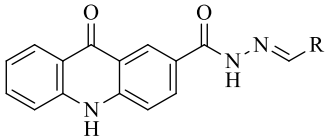


A1-A24



A25-A31

R = -H, CH_3, OCH_3, F, Cl, OH, NO_2

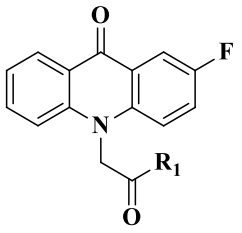


R= Furanyl & Pyridyl

A22 & A23

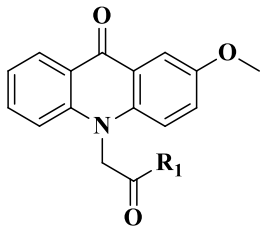
6.1.2 Chemistry of acridone -N-acetamides

To explore the cytotoxicity and AKT inhibitory activity of new acridone-*N*-actemides we tried to synthesize and evaluate them. Synthesis of acridone compounds involves the coupling of 2-chlorobenzoic acid and substituted anilines in the presence of copper catalyst followed by cyclization with polyphosphoric acid resulting in the formation of 2-fluoro/methoxy substituted acridones. Further *N*-alkylation with ethyl bromoacetate in the presence of DMF followed by ester hydrolysis furnished 2-fluoro/methoxy substituted acridone-*N*-acetic acid. The final derivatives were synthesized by coupling an acid derivative of acridone with different substituted amines (aromatic/aliphatic/heterocyclic) by using coupling reagents which generated two series, one with fluoro- (**AF-1-AF-10**) and the other with methoxy substitution (**AN-1-AN-10**).



AF-1-AF-10

R_1=

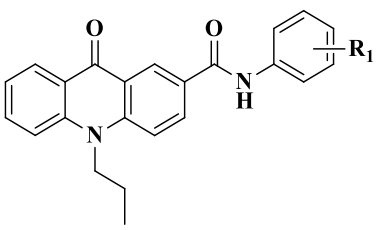


AN-1-AN-10

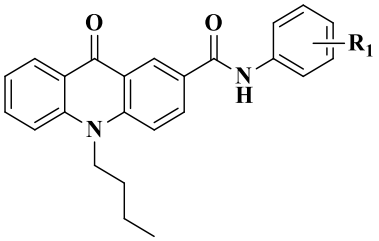
-morphonyl	$-NH(CH_2)_2N(CH_3)_2$	-NH-phenyl	-NH-4'-CH_3-phenyl
-piperidinyl	$-NH(CH_2)_2N(C_2H_5)_2$	-NH-4'-F-phenyl	-NH-4'-OCH_3-phenyl
-NH-thiazolyl	$-NH(CH_2)_2OCH_3$		

6.1.3 Chemistry of N-substituted Acridone-2-carboxamides

Acridone-2-carboxylic acid was synthesized as per explained method in scheme I and used as starting material, which was for further synthesis of target compounds. Then, N-alkylation was carried out with n-bromopropane/n-bromobutane in solvent DMF to get N^{10}-substituted acridone. The final compounds were synthesized by coupling acridone-2-carboxylic acid with different substituted aromatic amines in THF in the presence of TEA which yielded two series, one with *N*-propyl substituted acridone-2-carboxamides (**PA-1**-**PA-7**) and the other with *N*-butyl substituted acridone-2-carboxamides (**PB-1**-**PB-7**).



PA-1-PA-7



PB-1-PB-7

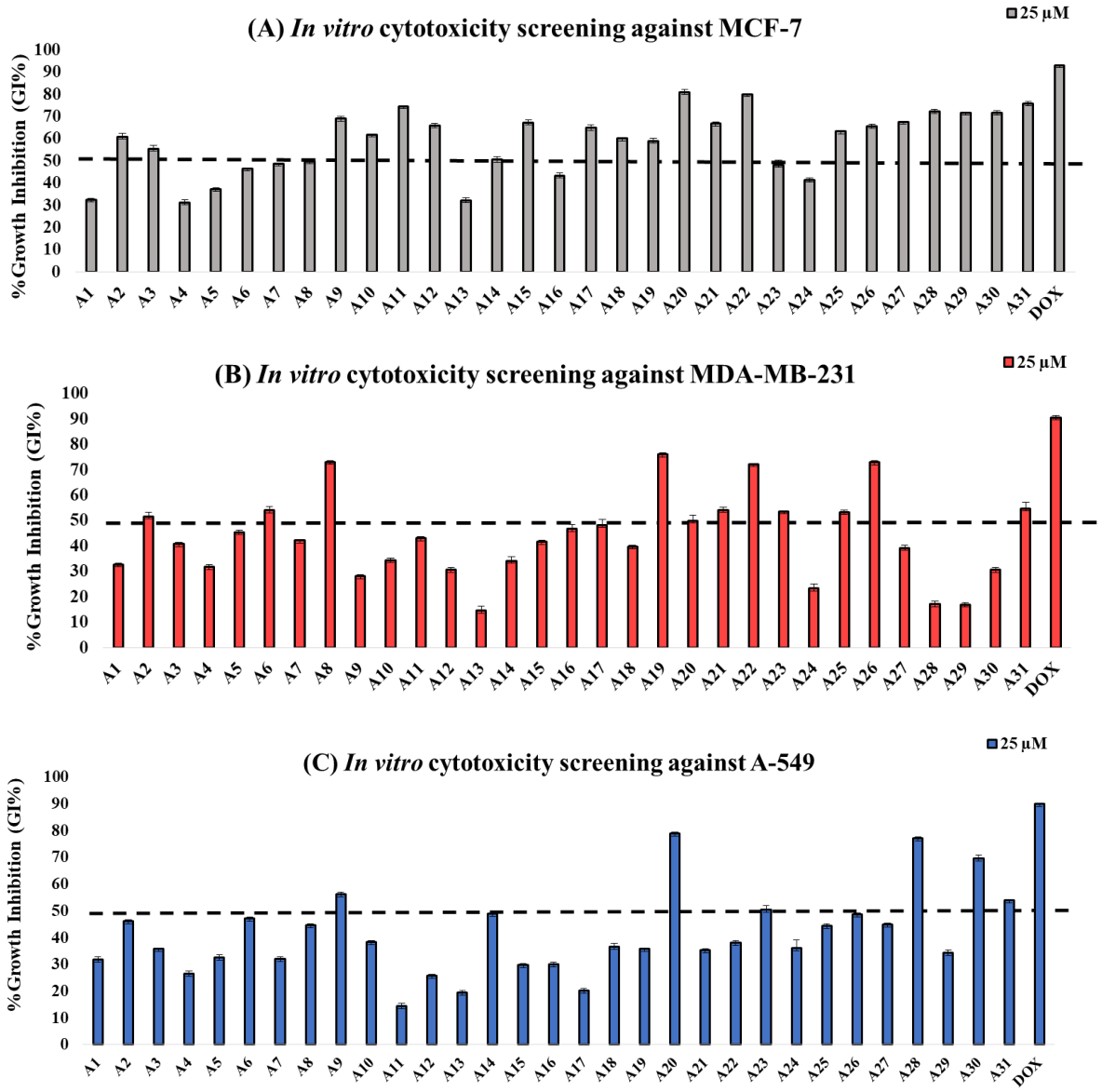
R_1= -H, -Me, -OMe, -F, -Cl, -NO_2, Azobenzene

6.2 *In vitro* cytotoxicity assay

6.2.1 *In vitro* cytotoxicity analysis of Acridone-2- carbohydrazide

All synthesized compounds (**Tables 1** and **2)** were subjected to *in vitro* anti-proliferative assessment against various cancer cell lines such as MCF-7, MDA-MB-231, A-549, and A-431. Acridone carbo-hydrazide derivatives were initially screened against these four cancer cell lines at a single dose of 25 µM concentration. Cells were treated with compounds for 72h and the mean percentage growth inhibition (GI %) of all compounds was calculated. All the synthesized compounds (**A1-A23)** and **(A24-A31**) showed moderate to excellent inhibitory activity against all tested cell lines in 25 µM except A-431. A total of 20 compounds displayed more than 50% growth inhibition against the MCF-7 cell line (**Fig. 5**). From them, compounds **A11**, **A12**, **A15**, **A17**, **A20**, **A21**, **A22**, and **A25-A31** exerted good to excellent GI% ranging from 63.18 to 80.89%. Similarly, tested compounds displayed moderate inhibitory activity against the MDA-MB-231 cell line. Compounds **A2**, **A6**, **A8**, **A19**, **A21**, **A22**, **A23**, **A25**, **A26** and **A31** more than 50% growth

inhibition. For the A-549 cell line, only five compounds **A20**, **A23**, **A28**, **A30,** and **A31** had moderate to good GI % range from 50.37 to 78.88. Regarding breast cancer cell lines, six compounds possess good inhibitory activity against MCF-7 whereas three compounds displayed good inhibition to the growth of the MDA-MB-231 cell line. All these compounds were selected and subjected to IC_{50} determination by using an SRB assay.


(A) In vitro cytotoxicity screening against MCF-7
25 µM
% Growth Inhibition (GI%)
(B) In vitro cytotoxicity screening against MDA-MB-231
25 µM
% Growth Inhibition (GI%)
(C) In vitro cytotoxicity screening against A-549
25 µM
% Growth Inhibition (GI%)
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12 A13 A14 A15 A16 A17 A18 A19 A20 A21 A22 A23 A24 A25 A26 A27 A28 A29 A30 A31 DOX

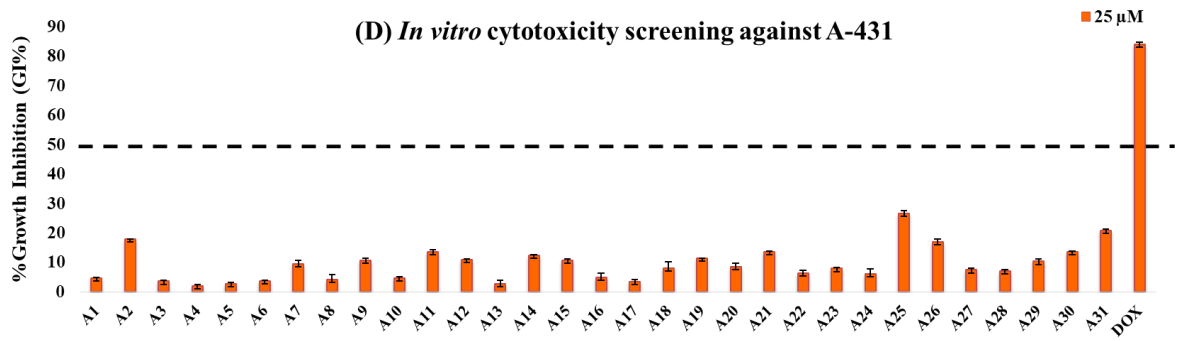


Figure 5: *In vitro* cytotoxicity screening of acridone -2-carbohydrazide derivative against A) MCF-7 B) MDA-MB-231 C) A-549 D) A-431

Table 5: *In vitro* cytotoxicity data in IC_{50} of synthesized compounds from Scheme 1

Sr. No.	Comp Code	IC_{50} (µM)[a] ± S.D.		
		MCF-7	MDA-MB-231	A-549
1	A2	15.64 ± 0.62	23.93 ± 2.04	39.42 ± 1.14
2	A3	17.82 ± 0.79	45.07 ± 1.50	47.18 ± 1.98
3	A8	28.32 ± 1.65	18.76 ± 0.68	35.46 ± 0.47
4	A9	10.55 ± 1.77	50.36 ± 0.49	19.65 ± 0.66
5	A10	18.73 ± 1.29	41.15 ± 0.46	45.34 ± 0.79
6	A11	7.92 ± 0.10	33.63 ± 2.23	68.96 ± 0.35
7	A12	22.63 ± 2.2	46.23 ± 0.77	51.74 ± 1.52
8	A14	27.36 ± 1.78	39.17 ± 1.42	34.82 ± 0.36
9	A15	32.21 ± 0.42	45.73 ± 0.92	49.43 ± 1.35
10	A17	20.93 ± 2.22	38.71 ± 0.23	54.38 ± 1.27
11	A18	21.83 ± 2.16	43.52 ± 0.18	37.38 ± 1.42
12	A19	37.62 ± 0.79	16.75 ± 0.62	38.92 ± 0.98
13	A20	8.39 ± 1.59	26.93 ± 2.04	11.32 ± 0.49
14	A21	17.40 ± 0.72	45.15 ± 1.65	38.45 ± 0.58
15	A22	6.24 ± 0.72	9.92 ± 0.10	34.82 ± 0.36
16	A23	38.59 ± 4.4	29.62 ± 0.12	25.93 ± 0.76
17	A25	13.68 ± 4.22	18.81 ± 0.81	29.84 ± 0.88
18	A26	14.47 ± 4.12	7.45 ± 0.17	27.36 ± 0.63
19	A27	15.29 ± 1.25	17.32 ± 1.76	31.11 ± 0.43
20	A28	10.39 ± 0.87	21.83 ± 2.16	8.52 ± 0.69
21	A29	12.86 ± 0.29	23.46 ± 0.43	36.47 ± 0.81
22	A30	18.69 ± 3.90	32.53 ± 1.45	13.90 ± 0.21

23	A31	9.60 ± 0.93	17.35 ± 1.38	23.03 ± 0.35
24	DOX[b]	1.28 ± 1.19	2.6 ± 1.08	4.76 ± 0.15

The *in vitro* cytotoxicity of synthesized compounds from scheme I revealed that most of the compounds displayed moderate to good cytotoxic activity against both breast cancer cell lines compared with the control drug doxorubicin, indicating that compounds containing the acridone carbohydrazide with pyridine heterocyclic structure were toxic to breast cancer cells. A few of the compounds showed a cytotoxic effect of a few compounds (**A11**, **A20**, **A22**, **A28** & **A31**) on MCF-7 cells that was much greater than that on MDA-MB-231 and A-431 cells. Among them, the most promising compound **A22** showed excellent activity against MCF-7 and MDA-MB-231 cells (7.24 ± 0.72 and 9.92 ± 0.10 µM, respectively (**Table 5**). Compound **A24** showed better activity (IC_{50} 7.45 ± 0.17 µM) against MDA-MB-231 than MCF-7 (IC_{50} 14.47 ± 4.12 µM).

Additionally, the cellular toxicity of the selected compounds was also evaluated using normal murine embryonic fibroblast (NIH/3T3) cell lines at final concertation of 50 µM to see if the molecules have specificity for cancer cells for their anti-proliferative effect. Their results indicated that these compounds had no significant effect on the growth of normal cells (**Fig. 6**).

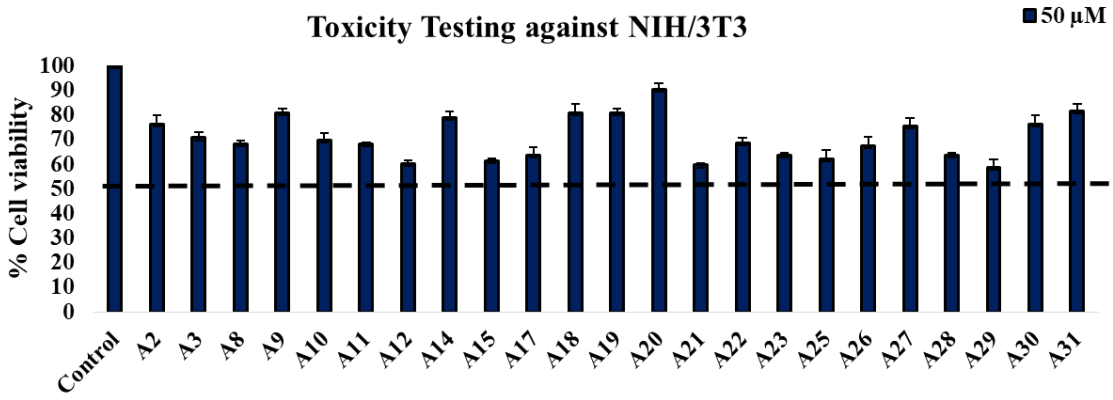


Figure 6: Toxicity testing of acridone-2carbohydrazide against NIH/3T3

6.2.2 Structure-Activity Relationship (SAR) of acridone-2-carbohydrazides

After the results of the cell proliferation assay and AKT enzyme inhibition studies, compounds **A1-A24** and **A25-A31** bearing the same acridone carbohydrazide moiety but with different substituents on the phenyl ring were subjected to SAR studies and few assumptions were made and summarized here (**Fig. 7**).

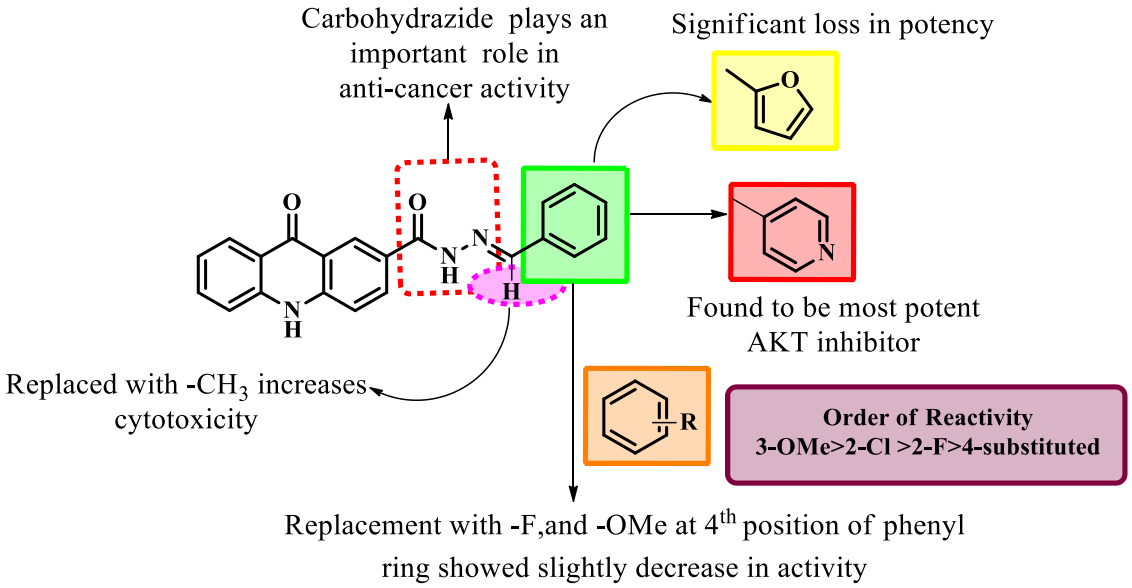

Carbohydrazide plays an important role in anti-cancer activity
Significant loss in potency
Found to be most potent AKT inhibitor
Replaced with -CH3 increases cytotoxicity
Order of Reactivity
3-OMe>2-Cl >2-F>4-substituted
Replacement with -F,and -OMe at 4th position of phenyl ring showed slightly decrease in activity

Figure 7: SAR of acridone-2-carbohydrazides

1. The phenyl ethylidene derivatives of acridone carbohydrazides were found to be more potent against MCF-7 and MDA-MB-231.
2. The presence of electron-withdrawing and donating substituents at the *o*-, *p*-, and *m*-positions of the phenyl ring play a significant role in cytotoxicity.
3. In benzylidene substituted derivatives, the substitution of the methoxy group at the 3rd position (**A11**) of the phenyl ring has improved the cytotoxicity against both the cell lines whereas the di-substitution of the methoxy group at the 3rd and 4th positions (**A13**) has remarkably reduced the cytotoxicity.
4. The cytotoxicity of the benzylidene acridone carbohydrazide moiety was significantly increased when it was substituted with an *m/p*-methoxy group (**A6** and **A11**), and reduced

with *p*-F (**A4**) and *p*-Cl (**A8**) groups. While the presence of –Cl, -OH, and -F at the ortho position enhanced cytotoxicity against MCF-7 cells.

5. When the aromatic ring of the benzylidene of acridone carbohydrazide was replaced with a heterocyclic ring such as furan, the resultant compound **A23** showed a significant loss in potency and cytotoxicity.
6. The introduction of a pyridine ring instead of furan in the structure resulted in compound **A22** found to be approx. 5-fold more potent than **A23** against breast cancer cell lines.
7. In the case of phenyl ethylidene substituted derivatives, di-substitution of halo group (-Cl) at the 2nd and 4th position (**A31**) has improved the cytotoxicity against both the cell lines as compared to mono substituted compound (**A29**).
8. The benzylidene of acridone carbohydrazide (**A4**, **A6**, **A7**, and **A8**) moiety replaced with phenyl ethylidene (**A28**, **A26**, **A29**, and **A27**) displayed IC_{50} less than 20 µM resulted in a significant increase in cytotoxicity suggesting that the presence of methyl group is needed to enhance the cytotoxic activity of the compound.

These results indicated that the electron-negativity, position, and steric effect in the benzene ring may change the cytotoxic profile of these compounds.

6.2.3 *In vitro* cytotoxicity analysis of Acridone-*N*-acetamides

The synthesized compounds (**AF1-AF10** and **AN1-AN10**) (**Fig. 5** and **Table 3**) were evaluated for *in vitro* cytotoxicity against a panel of human cancer cells lines such as A-549, A-431, MCF-7, and MDA-MB-231 and doxorubicin was used as the positive control. The effect of synthesized compounds on the viability of cells was determined and most of the compounds showed moderate to significant cytotoxicity against the above-mentioned cancer cell lines. Results obtained from cytotoxicity assay suggested that their cytotoxic activity was found to be potent in compounds **AF-9** and **AN-9**, moderate in compounds **AF-7**, **AF-8**, **AF-10**, **AN-7**, **AN-8,** and **AN-10** while less in compounds **AF-1-AF-6** and **AN-1-AN-6**. None of the compounds were found to be active against A-431.

Table 1: *In vitro* cytotoxicity data in IC_{50} of synthesized compounds from Scheme II

Sr. No.	Comp Code	IC_{50} (µM) (Average± S.D.)			
		MCF-7	MDA-MB-231	A-549	A-431

1	AF-1	41.63 ± 0.19	46.01 ± 2.91	43.06 ± 2.43	>100
2	AF-2	51.54 ± 2.36	60.86 ± 2.04	65.62 ± 2.65	>100
3	AF-3	53.24 ± 2.33	72.72 ± 3.06	68.92 ± 1.05	>100
4	AF-4	43.44 ± 3.16	48.50 ± 1.76	55.52 ± 1.76	>100
5	AF-5	42.53 ± 2.09	41.02 ± 1.51	36.81 ± 1.83	>100
6	AF-6	56.43 ± 0.89	58.72 ± 3.74	59.64 ± 1.68	>100
7	AF-7	18.40 ± 1.95	32.05 ± 0.39	20.68 ± 0.91	>100
8	AF-8	13.96 ± 2.22	24.23 ± 1.62	18.56 ± 0.36	>100
9	AF-9	8.25 ± 3.28	16.48 ± 2.93	15.39 ± 0.48	>100
10	AF-10	24.03 ± 2.96	32.56 ± 2.54	26.82 ± 0.21	>100
11	AN-1	35.26 ± 1.02	42.35 ± 1.69	41.29 ± 0.59	>100
12	AN-2	39.78 ± 2.36	45.52 ± 1.82	46.16 ± 1.59	>100
13	AN-3	46.89 ± 1.64	64.39 ± 1.19	53.07 ± 2.96	>100
14	AN-4	37.65 ± 1.12	42.08 ± 2.18	51.06 ± 2.15	>100
15	AN-5	35.58 ± 0.68	38.76 ± 1.65	32.06 ± 1.08	>100
16	AN-6	49.43 ± 0.29	54.25 ± 2.95	53.65 ± 0.62	>100
17	AN-7	16.04 ± 2.56	27.73 ± 2.36	24.62 ± 0.21	>100
18	AN-8	9.45 ± 1.08	21.58 ± 2.36	16.23 ± 0.36	>100
19	AN-9	6.76 ± 2.49	12.69 ± 2.36	9.59 ± 0.49	>100
20	AN-10	20.75 ± 0.68	30.48 ± 1.83	23.76 ± 3.12	>100
21	DOX	1.28 ± 1.19	2.6± 1.08	4.76 ± 0.15	10.56 ± 1.62

From **Table 6** it was observed that few compounds exhibited good cytotoxicity against tested cell lines. Among them, 4-fluoro derivative (**AN-8**, IC_{50}= 9.45 µM), 4-methyl derivative (**AN-9**, IC_{50}= 6.76 µM) from 2-methoxy substituted acridones, and 4-methyl derivative (**AF-9**, IC_{50}= 8.25 µM) from 2-fluoro substituted acridones exhibited good cytotoxicity against MCF-7 with IC_{50} less than 10 µM. Only 4-methyl derivatives (**AN-9**, IC_{50}= 9.59 µM, and 12.69 µM) displayed good activity against A-549 and MDA-MB-231 respectively. In comparison, the title compounds **AF-9**, and **AN-9** have shown appreciable cytotoxicity against MCF7, A549, and MDA-MB-231 cell lines.

Apart from the efficacy of compounds in suppressing the viability of lung and breast cancer cells, the cytotoxic effect of these compounds on normal cell lines (NIH/3T3) was also assessed at 50 µM. There was no significant effect on the growth of normal cells. Hence, we can say that none of the compounds were found to be toxic to the normal cells (**Fig. 8**).

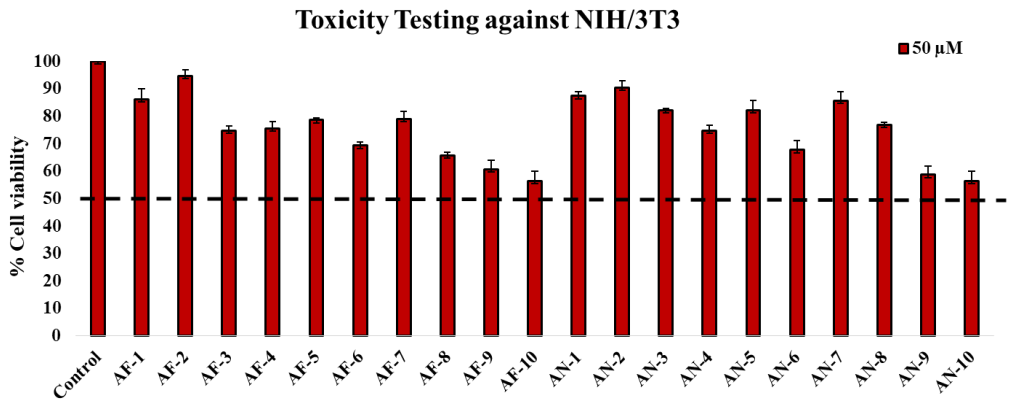


Figure 8: Toxicity testing of acridone-*N*-acetamides against NIH/3T3

6.2.4 SAR of Acridone-*N*-acetamides

From the anti-proliferative and *in vitro* kinase assay, the following conclusions were made about the SAR, and have been described here (**Fig. 9**).

1. Acridone-*N*-acetamides substituted with phenyl ring exhibited good cytotoxicity against A-549, MCF-7, and MDA-MB-231 except for A-431.
2. The presence of the methoxy group at the C-2 position of the acridone ring displayed better anti-cancer activity as compared to the fluoro group at the same position.
3. The amide linker between the acridone ring and aromatic ring played an important role in the bioactivity.
4. The introduction of a methyl group at the C4' position of the phenyl ring was found to be more cytotoxic as compared with other derivatives.
5. Changing the methyl group to fluoro or methoxyl group showed a decrease in anti-proliferative activity.
6. Replacement of the phenylamino group with aliphatic amines such as *N*, *N*-dimethylethylamine, *N*, *N*-dimethylethylamine, and 2-methoxyethylamine displayed 5-fold lesser activity as compared with **AN-8**.
7. Moreover, the introduction of heterocyclic amines like morpholine and piperidine resulted in a significant loss in anti-cancer activity, especially in compounds **AN-5** and **AN-6**.
8. Surprisingly, compound **AN-7** with no substituents on the phenyl ring displayed better anti-cancer activity than 4-methoxy substituted phenyl ring **AN-10**.

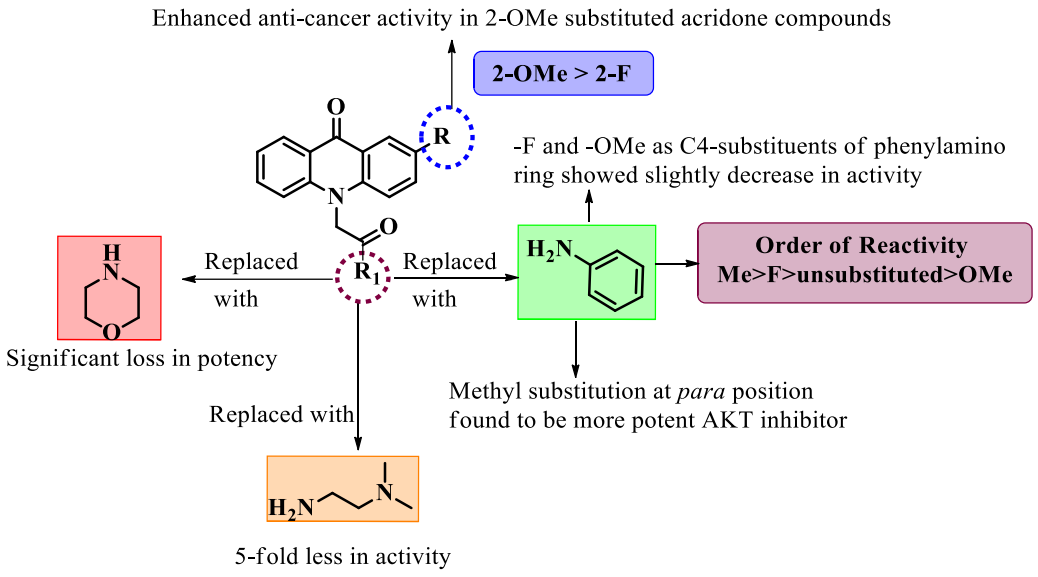

Enhanced anti-cancer activity in 2-OMe substituted acridone compounds
2-OMe > 2-F
-F and -OMe as C4-substituents of phenylamino ring showed slightly decrease in activity
Order of Reactivity
Me>F>unsubstituted>OMe
Replaced with
Replaced with
Significant loss in potency
Methyl substitution at *para* position found to be more potent AKT inhibitor
Replaced with
5-fold less in activity

Figure 9: SAR of Acridone-N-acetamides

These results indicated that the position, electron-negativity, and steric effect in the benzene ring might change the cytotoxic profile of compounds. The data help us to conclude that acridone-*N*-acetamides with aryl substitution favor anticancer activity preferably with methyl substitution on the phenyl ring at the 4th position. Also, 2- methoxy substitution on the acridone ring was found more effective than 2-fluoro substitution.

6.2.5 *In vitro* cytotoxicity evaluation of Acridone-2-carboxamides

The anti-proliferative activity of the synthesized N^{10}-substituted acridone derivatives (**Table 4**) having a carboxamide group at the 2nd position was determined using SRB assay, and Doxorubicin was used as the positive control. The synthesized compounds (**PA-1-PA-7**, and **PB-1-PB-7**) were screened for *in vitro* cytotoxicity against MCF7 and MDA-MB-231. The results of the *in vitro* cytotoxicity are expressed in terms of the compound concentration required to inhibit the growth of the cells by 50% with respect to the control (IC_{50} µM). The data from **Table 7**, showed that some of the acridone molecules displayed good anti-proliferative activity with low IC_{50} values.

Among the synthesized series *N*-butyl derivative of acridone showed significant cytotoxicity. Results obtained from the biological study revealed that compounds **PB-4**, **PB-5**, and **PB-6** were found to be active against both the breast cancer cell lines (MCF-7 and MDA-MB-231) with cytotoxicity of less than 10 µM. The compounds **PB-2**, **PB-3**, and **PB-7** showed moderate to good

cytotoxicity in the range of 12-30 µM against tested cell lines. *N*-propyl substituted series compounds **PA-4**, and **PA-6** exhibited better cytotoxicity against MCF-7 with IC_{50} less than 10 µM, whereas compound **7f** displayed good cytotoxicity against MDA-MB-231 with an IC_{50} of 6.14 µM. The compounds **PA-2**, **PA-5**, and **PA-7** showed cytotoxicity in the range of 20-30 µM against the MCF-7 breast cancer cell line. The structure-activity relationship suggested that the substitution of the methoxy group and azo-benzene on the phenyl ring lowered the cytotoxicity against both cell lines.

Table 7: *In vitro* cytotoxicity data in IC_{50} of synthesized compounds from Scheme III

Sr. No.	Comp Code	IC_{50} (µM) (Average± S.D.)	
		MCF-7	MDA-MB-231
1	PA-1	56.14 ± 4.12	67.19 ± 1.12
2	PA-2	27.7± 3.15	31.8 ± 2.61
3	PA-3	36.82 ± 2.16	34.32 ± 3.16
4	PA-4	8.56 ± 0.57	10.58± 1.01
5	PA-5	10.54 ± 1.77	13.68 ± 4.26
6	PA-6	5.41 ± 0.07	6.14± 0.72
7	PA-7	28.26 ± 0.27	29.14 ± 1.68
8	PB-1	41.63 ± 0.19	56.68 ± 3.65
9	PB-2	23.7 ± 2.12	26.2 ± 2.18
10	PB-3	20.16 ± 1.77	19.68± 0.93
11	PB-4	4.90 ± 0.11	9.16 ± 4.64
12	PB-5	7.24 ± 2.56	9.60 ± 0.93
13	PB-6	4.72 ± 0.15	5.53 ± 0.65
14	PB-7	12.63± 2.27	19.14 ± 4.64
15	DOX	1.28 ± 1.19	2.6± 1.08

In addition, the cellular toxicity of the synthesized compound was again evaluated against normal NIH/3T3 cells, results revealed that none of the compounds showed a significant effect on the growth of normal cells (**Fig. 10**).

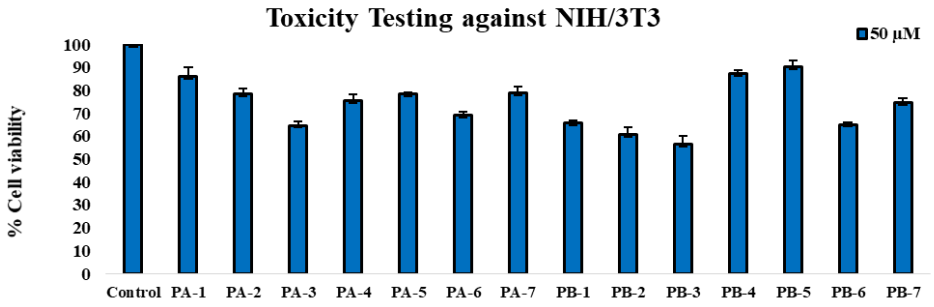


Figure 10: Toxicity testing of acridone-2-carboxamides against NIH/3T3

6.2.6 SAR of N-substituted Acridone-2-carboxamides

Based on *in vitro* cytotoxicity and AKT inhibitory activity of all the synthesized N^{10}-substituted acridone-2-carboxamides against breast cancer cell lines, the following assumptions about the SAR have been clearly described (**Fig. 11**).

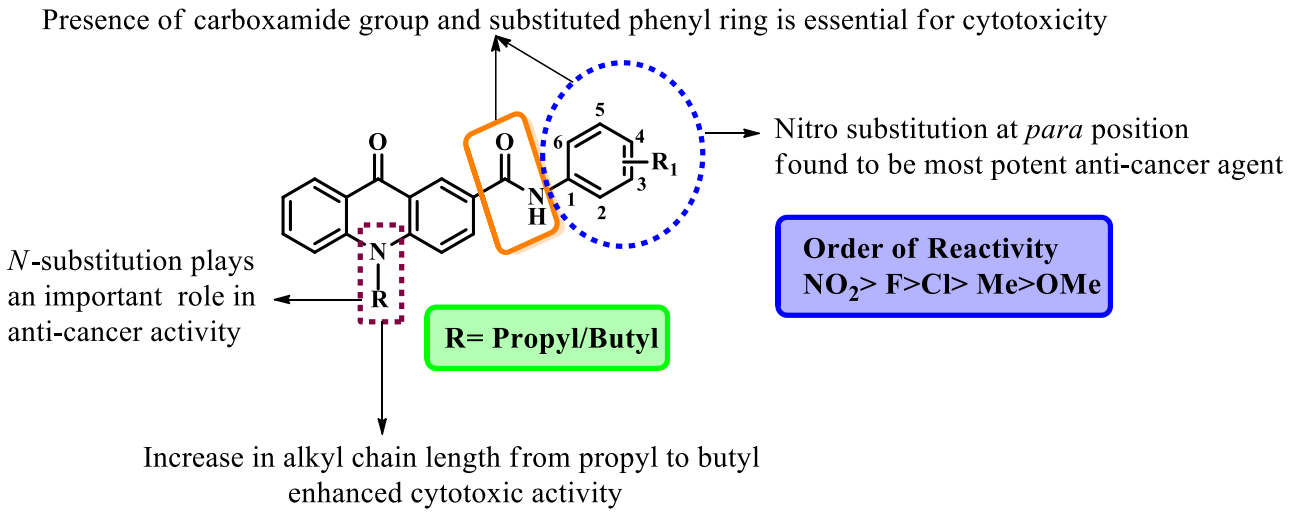

Presence of carboxamide group and substituted phenyl ring is essential for cytotoxicity
Nitro substitution at *para* position found to be most potent anti-cancer agent
Order of Reactivity
NO2> F>Cl> Me>OMe
N-substitution plays an important role in anti-cancer activity
R= Propyl/Butyl
Increase in alkyl chain length from propyl to butyl enhanced cytotoxic activity

Figure 11: SAR of N-substituted Acridone-2-carboxamides

1) The presence of the carboxamide group at the 2nd position and alkyl substitution at the N^{10} position of the acridone ring is very important for anti-cancer activity.
2) *N*-butyl-substituted acridone-2-carboxamides were found more active than *N*-propyl-substituted acridone-2-carboxamides.

3) An increase in the length of the alkyl chain from propyl to butyl at the N^{10} position of acridone showed significant enhancement in the anti-cancer activity.
4) Compound with the substituted nitro group at the 4th position phenyl ring showed more potent anti-cancer activity in both MCF-7 and MDA-MB-231 cell lines.
5) Compound with substitution of nitro, fluoro, and chloro group on phenyl at 4th position showed more potent anti-cancer along with AKT inhibitory activity.
6) The replacement of the 4-chlorophenyl with 4-fluorophenyl showed a remarkable increase in the cytotoxicity of both MCF-7 and MDA-MB-231 cell lines.
7) The Carboxamide linker attached between the phenyl ring and acridone ring showed a rise in activity against both the cell lines.
8) The derivative of 4-methoxyphenyl and 4-methyl phenyl have less activity compared to 4-chlorophenyl, 4-fluorophenyl, and 4-nitrophenyl derivatives.
9) Among the different *para* substituents, the order of activity was found to be NO_2 >F > Cl > CH_3> OCH_3.
10) Although derivatives with a substituent such as azo-benzene at the 4th position on the phenyl ring have shown moderate anti-cancer activity, they showed potent AKT inhibitory activity

These results indicate that the position of substituents, electron-negativity, and steric effect in the benzene ring might change the cytotoxic profile of acridone compounds.

Further, all promising compounds from schemes I, II, and III were evaluated for AKT kinase inhibitory activity assay

6.3 *In-vitro* AKT kinase inhibition assay:

As per the *in vitro* cytotoxicity assay results, compounds (**A11**, **A20**, **A22, A28,** and **A31**) from **scheme I,** compounds (**AF-8**, **AF-9**, **AN-8,** and **AN-9**) from **scheme II,** and compounds (**PA-4**, **PA-6**, **PB-4, PB-5,** and **PB-6**) had good anti-cancer activity against MCF-7 (Akt over-expressing) cells, that's why they were selected to screen by *in vitro* kinase inhibition assay to evaluate whether they can truly inhibit AKT kinase activity[30]. AKT kinase inhibitory activity of these compounds was evaluated by using compound **AC34 (**reported AKT inhibitor, **Fig. 1**) as a reference standard

having IC_{50} value 0.57 ± 0.76 µM, and then IC_{50} values of potential molecules were determined and presented in **Table 8**.

Table 8: IC_{50} determination of promising compounds from schemes I, II, and III

Comp. code	IC_{50} (µM)[a] (Average± S.D.)	Comp. code	IC_{50} (µM) [a] (Average± S.D.)
A11	5.10 ± 0.05	AN-8	8.74 ± 0.45
A20	7.36 ± 0.58	AN-9	3.25 ± 0.03
A22	1.75 ± 0.23	PA-4	15.32 ± 0.66
A28	8.98 ± 0.36	PA-6	6.90 ± 0.23
A31	2.40 ± 0.15	PB-4	11.96 ± 0.02
AF-8	10.21 ± 0.23	PB-5	10.35 ± 0.12
AF-9	5.36 ± 0.18	PB-6	5.38 ± 0.13
AC34[b]	0.57 ± 0.76	-	-

[a]The values are the mean ± SD of at least three independent experiments [b]AC34 = Reference standard

All the tested compounds from scheme I showed good AKT inhibitory activity with $IC_{50} < 10$ µM. Among them, **A22** and **A31** were found to be the most potent Akt inhibitors with IC_{50} 1.75 ± 0.23, and 2.40 ± 0.15 respectively. Compounds **AF-9** and **AN-9** from scheme II showed excellent AKT inhibitory activity at IC_{50} 5.36 ± 0.18, and 3.25 ± 0.03 respectively.
Compounds from Scheme III, **PA-6,** and **PB-6** were found to be potent AKT inhibitors with IC_{50} 6.90 ± 0.23 and 5.38 ± 0.13 respectively.

6.4 Quantitative estimation of p-AKT Ser^{473} and total-AKT

As *in-vitro* AKT kinase assay compounds **A11**, **A22,** and **A31** have the inhibitory potential towards AKT kinase in a dose-dependent manner, they were chosen for elucidating the mechanism behind the good AKT inhibition. Hence, a quantitative determination of the phosphorylated and total AKT levels was done to evaluate the inhibitory effect of these compounds in MCF-7 cells[32].

These compounds effectively inhibited the activation of p-AKT Ser^{473} in MCF-7 cells at 5 µM. Quantitative ELISA confirmed the above result and showed that these compounds significantly reduced the levels of p-AKT Ser^{473} in MCF-7 cells (**Fig. 12**). Among them, compound **A22**

displayed a significant reduction in levels of p-AKT Ser^{473} in comparison to total AKT at all the tested concentrations, while compounds **A11** and **A31** showed a significant reduction of p-AKT at higher concentrations only. These same compounds produced a minimal inhibitory effect on the levels of total AKT at tested concentrations. Furthermore, these results suggested that the inhibition of p-AKT Ser^{473} and total AKT might be responsible for the good activities of compound **A22** against MCF-7 cell lines. From the results, we can conclude that compounds (**A11, A22,** and **A31)** were found to have potential anti-cancer nature due to p-AKT Ser^{473} inhibition.

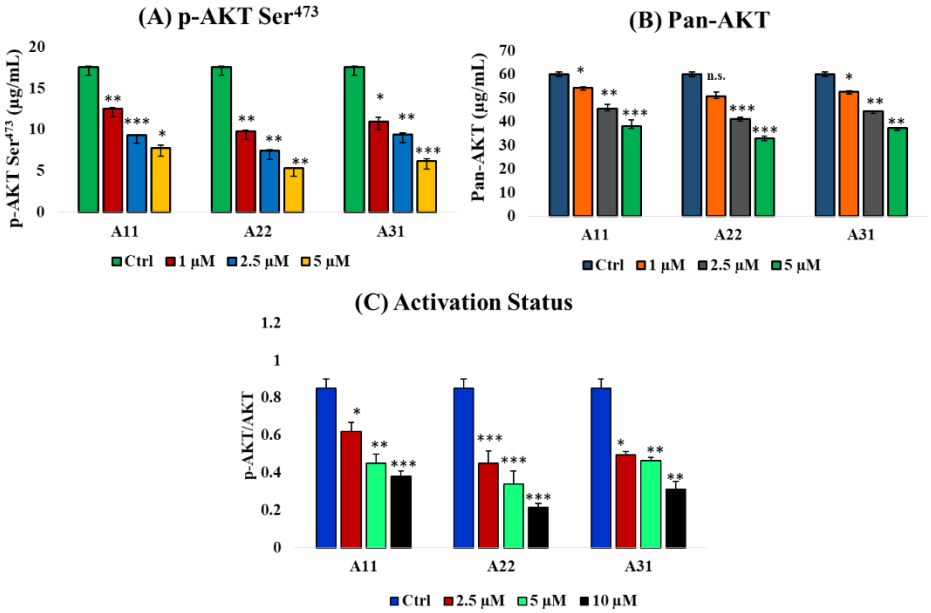


Figure 12: The MCF-7 cells were treated with compounds at different concentrations (0, 1, 2.5, and 5 µM) for 24 h. Quantitative ELISA assay results of compounds of **A11**, **A22**, and **A31** on (A)Levels of p-AKT Ser473 (B) Levels of Total AKT (C) The activation of p-AKT Ser473 evaluated by quantitative ELISA assay. A significant difference in p-AKT Ser473 was indicated between the control group and the **A22** treated group. n.s, no significant difference, * P < 0.05, **P < 0.01, ***P < 0.001. Error bars, mean ±SD of three independent experiments. Compounds inhibit cancer cell growth by suppressing the activation of AKT signaling.

6.5 Cell Cycle Analysis:

Compounds **A11, A22,** and **A31** from scheme I and **AF-9** and **AN-9** from scheme II were chosen for elucidating the antitumor mechanism responsible for anti-proliferation. We evaluated their effect on the cell cycle. MCF-7 cell line was treated with selected compounds for 24 h[35]. The DNA content in samples was analyzed by using a FAC scan Flow cytometer at IIT Powai.

Table 9: Cell cycle Analysis Data of acridone compounds

Cell Population	Control cells (%)	A11 (%)	A22 (%)	A31 (%)	AF-9 (%)	AN-9 (%)
Sub-G1	4.01	3.01	3.9	4.29	6.68	4.05
G0/G1	**62.5**	**67.3**	**69.1**	**65.9**	**68.5**	**70.5**
S	20.8	17.7	17.7	19.8	15.3	16.7
G2/M	11.0	11.4	8.27	8.91	8.84	7.58

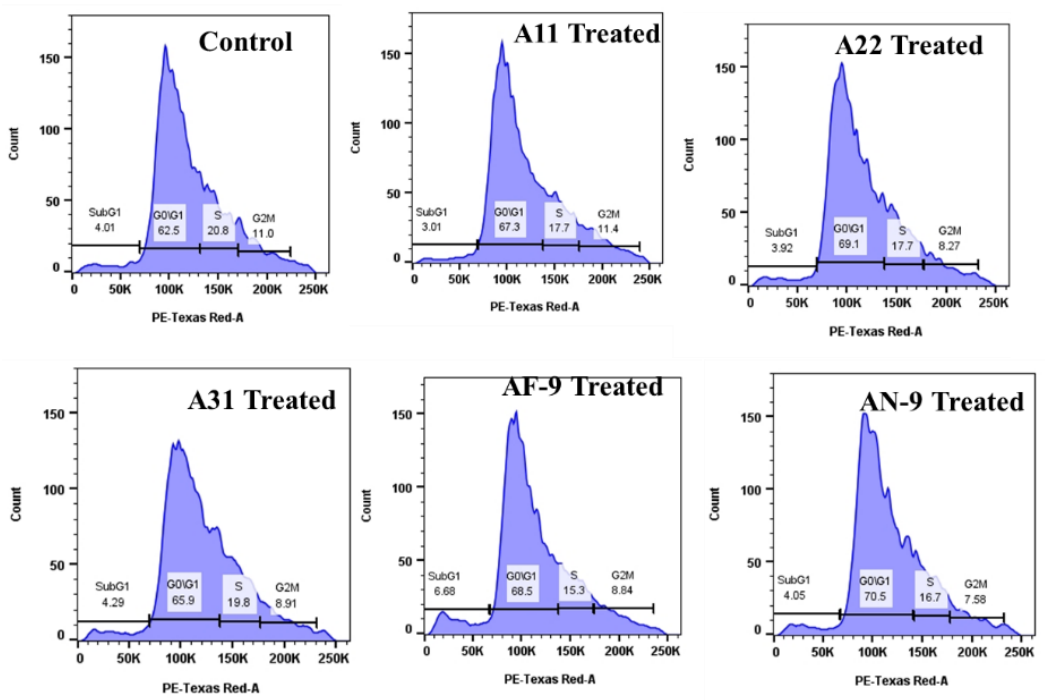


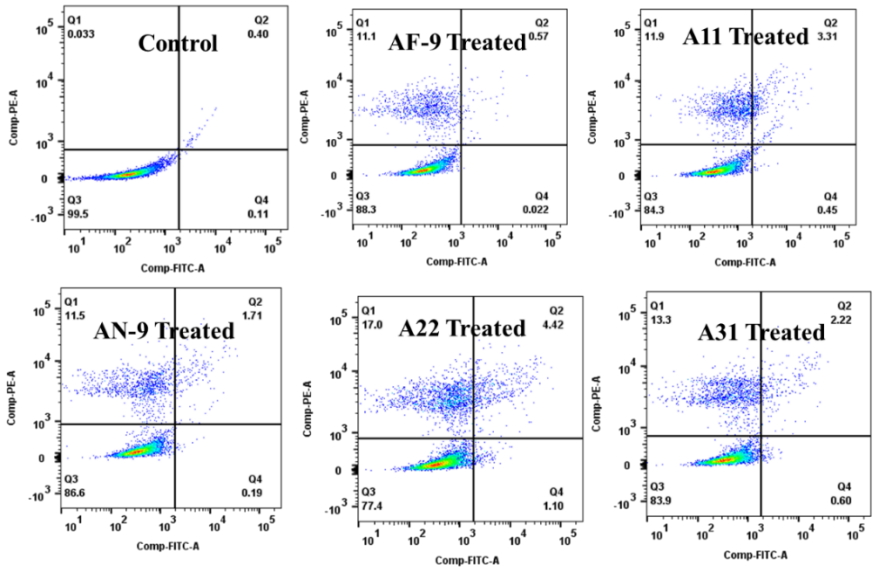
Figure 13: Cell Cycle profiles of compounds

The obtained results were compared with the non-treated MCF-7 cell line as a blank control (**Fig. 13** and **Table 9**). The percentage of G0/G1-phase cells increased from 62.5 % (blank control) to 69.1 %, 68.5%, and 70.5% at their IC_{50} for compounds **A22, AF-9, and AN-9** respectively. These results indicated that the compounds **A22** and **AN-9** significantly caused cell cycle arrest in G0/G1-phase in the MCF-7 cell line.

6.6 Cell Apoptosis Analysis

To enumerate the % of cells undergoing apoptosis with the treatment of acridone derivatives apoptosis assay was performed. Here, the apoptotic activity of the compounds (**A11**, **A22**, **A31**,

AF-9, and **AN-9**) was assayed using Annexin V-FITC/PI double staining method. Cells that are propidium iodide (PI) negative and Annexin V positive are expressed in Q2 and Q4 and are reflected as apoptotic cells (**Fig. 14**). Q2 and Q4 are early and late apoptotic cells quadrant. The cells that are both (PI and Annexin V) are expressed in Q1 (necrotic cells) and both negative to (PI and Annexin V) are expressed in Q3 (living cells)[36].



Comp. code	% Apoptotic cells		
	Early	Late	Total
Control	**0.40**	**0.11**	**0.51**
AF-9	0.57	0.022	0.592
AN-9	1.71	0.19	1.90
A11	**3.31**	**0.45**	**3.75**
A22	**4.42**	**1.10**	**5.52**
A31	2.22	0.60	2.82

Figure 14: Cell Apoptosis analysis of compounds

The obtained results were compared with the untreated MCF-7 cell line as control and the values of total apoptosis (early and late apoptotic cells, Q2 + Q4) were calculated. For compound **A22**, the total apoptotic cells % significantly increased from 0.51 % to 5.52 % which was higher than **A11** and **A31** at corresponding concentrations. For compounds **A11** and **A31**, the total apoptotic cells % was found to be 3.75 % and 2.82 % respectively. From the data represented in **Fig. 14**, it is indicated that these compounds, (**A11, A22,** and **A31)** showed an increased the % of annexin V apoptotic cells in both the early and late stages. These compounds also showed an increase in % of necrotic cells that might be due to external environmental factors such as metabolic stresses including hypoxia. Among them, compound **A22** displayed a significant 10-fold increase in apoptotic cells as compared to the control. This study revealed that **A22** displayed cytotoxicity against the MCF-7 cell line through apoptosis induction.

6.7 *In silico* Studies

6.7.1 Molecular Docking

To understand the binding pattern of the synthesized compounds molecular docking studies were performed with AKT (PDB ID: 4GV1). Autodock Vina software was used to analyze the binding modes of acridone compounds in their active site of the selected target proteins. Enzyme-ligand complexes were analyzed by docking score and their orientation in the active site of the enzyme structure[39]. Further analysis of results from molecular docking revealed that all the tested compounds showed good binding energy with AKT and It was ranging from -9.0 to -10.7 Kcal/mol. The 2D-docking interactions of the most potent compounds are depicted in **Fig. 15**.

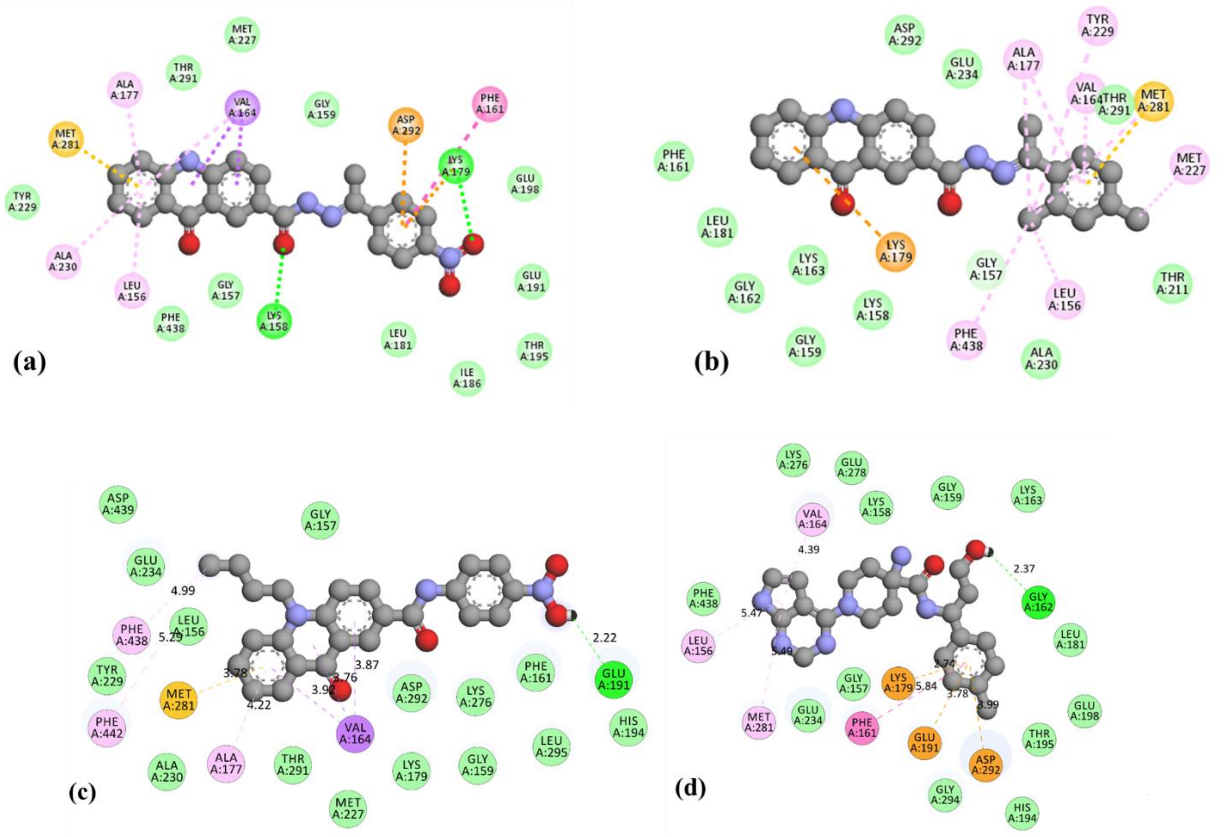


Figure 15:2D representation of binding interactions of acridone molecules (a) compound **A30**, (b) compound A**31**, (c) compound **PB-6** (d) Reference compound (AZD5363) with AKT1 (PDB: 4GV1).

6.7.2 *In silico* ADMET Prediction

In drug development and discovery, the prediction of the pharmacokinetic properties of any new compounds is an important criteria. Hence, ADMET and pharmacokinetic properties were

calculated using the online software tool SWISS-ADME to predict their drug-like nature. The drug compounds must obey the Lipinski rule to be developed as orally effective drugs. Any violation of this rule might lead to problems with pharmacokinetic properties[43].

As per the results mentioned in **Table 10**, all the subjected compounds displayed a molecular weight below 500 which indicates that they can be easily transported. The hydrogen bond acceptor (< 10) and donor (< 5) were found to be in the range of 3-4 and 0-2, respectively. Title compounds showed log P values in the range of 2.68–4.69 which is less than 5 this indicates the compounds exhibited good permeability. All the compounds showed high GI absorption and no blood-brain barrier permeation.

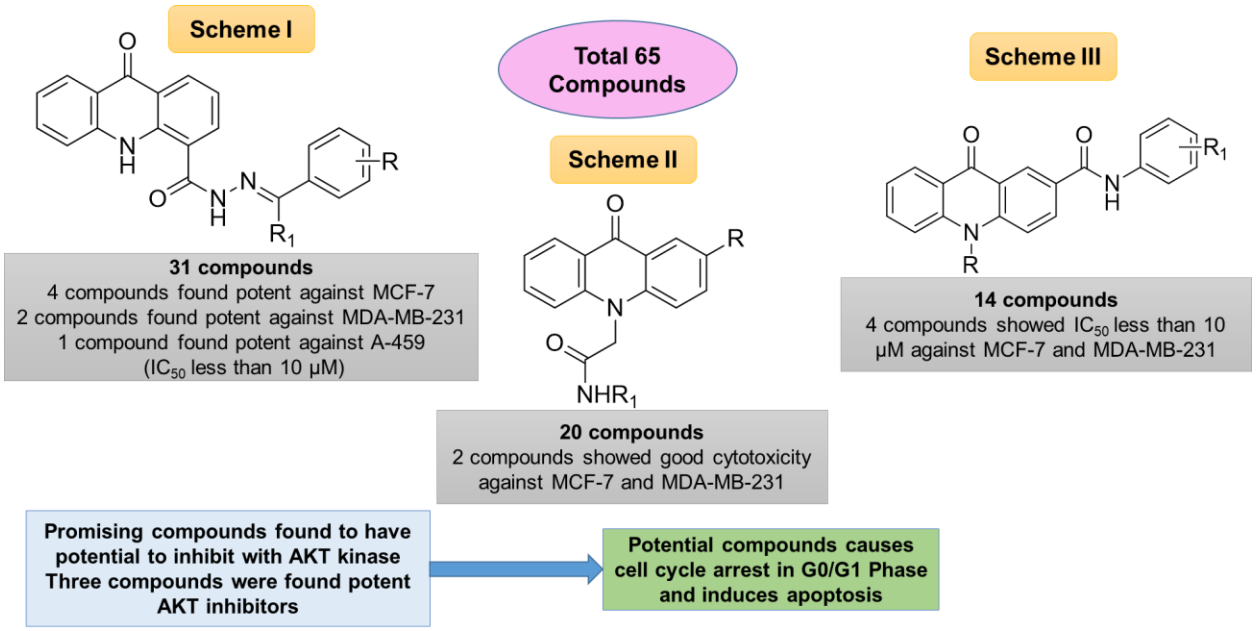
Table 2: Data obtained from predicted ADMET parameters

Descriptors	**Compound Codes**						
	A11	**A22**	**A31**	**AF-9**	**AN-9**	**PA-6**	**PB-6**
MW (g/mol)	371.39	342.35	424.28	360.13	372.15	401.41	415.44
No. of HBA	4	4	3	3	3	4	4
No. of HBD	2	2	2	1	1	1	1
TPSA	87.89	91.55	79	51	60	96.92	96.92
Consensus Log P	3.39	2.68	4.69	3.93	3.57	3.39	3.7
ESOL Log S	-4.7	-3.97	-6.02	-5.18	-5.09	-5.37	-5.6
GI Absorption	High	High	High	High	High	High	High
ESOL Class	MS	Soluble	PS	MS	MS	MS	MS
BBB permeation	No	No	No	Yes	Yes	No	No
BA score	0.55	0.55	0.55	0.55	0.55	0.55	0.55
Lipinski violations	0	0	0	0	0	0	0
LD_{50} mg/kg	1580.4	1119.5	2381.2	1528.1	1000.5	2103.4	2100.6

MW = Molecular weight; HBA= Hydrogen Bond Acceptor; HBD= Hydrogen Bond Donor; TPSA = Topological polar surface area; Log P = Logarithm of the partition coefficient; ESOL Log S = ESOL model logarithm of molar solubility in water; ESOL class = Solubility class in Log S scale; MS = Moderately soluble; PS = Poorly soluble; GI = Gastrointestinal; BBB = Blood–brain barrier; BA= Bioavailability

Results from *in silico* pharmacokinetic data revealed that all the compounds follow the Lipinski rule with no violation. These compounds have desired pharmacokinetics properties and can be considered as 'drug-like' for further development of a lead molecule.

7. Summary and Conclusions


Scheme I
31 compounds
4 compounds found potent against MCF-7
2 compounds found potent against MDA-MB-231
1 compound found potent against A-459
(IC50 less than 10 µM)
Total 65 Compounds
Scheme II
20 compounds
2 compounds showed good cytotoxicity against MCF-7 and MDA-MB-231
Scheme III
14 compounds
4 compounds showed IC50 less than 10 µM against MCF-7 and MDA-MB-231
Promising compounds found to have potential to inhibit with AKT kinase Three compounds were found potent AKT inhibitors
Potential compounds causes cell cycle arrest in G0/G1 Phase and induces apoptosis

- Compounds **A22, AN-9, and PB-6** showed potent cytotoxicity against a panel of cancer cell lines.
- **None of the tested compounds** were found to be **toxic** against normal fibroblast cell line such as NIH/3T3.
- Compounds (**A11**, **A22**, **A31**, **AF-9**, **AN-9**, and **PB-6**) displayed promising exhibit promising AKT kinase inhibitory activity.
- Compound **A22** can be considered an effective kinase inhibitor because of its potent AKT inhibitory activity.
- Compound **A22** significantly reduced the levels of p-AKT Ser^{473} than total AKT in a dose-dependent manner.
- Arrested cell cycle **at G0/G1** phase and induced apoptosis.
- The binding modes of compounds were similar to reference inhibitor and *in silico* ADMET studies of compounds predicted that they are suitable for oral administration
- The study recommended that compound **A22** can be considered a lead compound and exploited further to design and develop safer and more potent anticancer agents targeting AKT kinase.

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Candidate	**Supervisor**
Ms. Tanuja T. Yadav	**Dr. Mayur C. Yergeri**